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Biofilm Eradication Kinetics of the Ultrashort Lipopeptide C₁₂-OOWW-NH₂ utilizing a Modified MBEC assay™

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Keywords: Lipopeptides, Kill kinetics, Antimicrobial, Biofilm, MBEC

Abstract

In this study we report the antimicrobial planktonic and biofilm kill kinetics of ultrashort cationic lipopeptides previously demonstrated by our group to have a minimum biofilm eradication concentration (MBEC) in the microgram per mL ($\mu\text{g/mL}$) range against clinically relevant biofilm forming microorganisms. We compare the rate of kill for the most potent of these lipopeptides, dodecanoic (lauric) acid conjugated $\text{C}_{12}\text{-Orn-Orn-Trp-Trp-NH}_2$ against the tetrapeptide amide $\text{H-Orn-Orn-Trp-Trp-NH}_2$ motif and the amphibian peptide Maximin-4 via a modification of the MBEC AssayTM for Physiology & Genetics (P&G). Improved antimicrobial activity is achieved upon N-terminal lipidation of the tetrapeptide amide. Increased antimicrobial potency was demonstrated against both planktonic and biofilm forms of Gram-positive microorganisms. We hypothesize rapid kill to be achieved by targeting of microbial membranes. Complete kill against established 24 hour Gram-positive biofilms occurred within 4 hours of exposure to $\text{C}_{12}\text{-OOWW-NH}_2$ at MBEC values [methicillin-resistant *Staphylococcus epidermidis* (ATCC 35984): $15.63\mu\text{g/mL}$] close to the values for the planktonic minimum inhibitory concentration (MIC) [methicillin-resistant *Staphylococcus epidermidis* (ATCC 35984): $1.95\mu\text{g/mL}$]. Such rapid kill, especially against sessile biofilm forms, is indicative of a reduction in the likelihood of resistant strains developing with the potential for quicker resolution of pathogenic infection. Ultrashort antimicrobial lipopeptides have high potential as antimicrobial therapy.

Introduction

Cationic antimicrobial peptides exist throughout nature as defense mechanisms in both prokaryotic and eukaryotic organisms. Antimicrobial peptides have evolved over millennia to become inherent antimicrobial molecules and effective mediators of the innate and adaptive immune response (1). They have been proven to be effective at neutralizing Gram-negative bacterial lipopolysaccharide endotoxin and aid the process of wound healing (2,3). Varying in the length of their primary sequence, frequently ranging from 12 to 100 amino acids cationic variants possess an overall charge between +2 and +9. Cationic antimicrobial peptides display a reduced risk of developing resistance due to their multiple modes of action. These include targeting of both negatively charged bacterial membranes and intracellular processes including: mRNA and protein synthesis; DNA replication and protein folding (4,5). They show preferential binding for the negatively charged phospholipid bilayer of bacterial cells compared with mammalian membranes which are neutral due to the large presence of sterols, ergosterol (in fungi) and cholesterol (in mammalian cells) (4).

Amphibians represent one of the most studied sources of antimicrobial peptides in nature. Present in amphibian skin secretions at high concentrations, structural elucidation has made it possible to determine their structure for synthesis in the laboratory via solid phase peptide synthesis (6). Lai and colleagues demonstrated that the frog species *Bombina maxima* produced a group of basic peptides, called Maximins, which possessed minimum inhibitory concentrations (MIC) in the $\mu\text{g/mL}$ range against a broad spectrum of Gram-positive and -negative bacteria. The most potent of these, Maximin-4, consists of twenty seven amino acids (GIGGVLLSAGKAALKGLAKVLAEKYAN) with an MIC of $2.7\mu\text{g/mL}$ against *Staphylococcus aureus* (*S.aureus*) (7).

Of particular interest in modern healthcare is the development of cost-effective ultrashort cationic lipopeptides by rational design of the peptide motif. This involves application of structural activity relationships whereby an optimum hydrophobic: charge balance is achieved via sequential conjugation of saturated fatty acids to a minimum peptide pharmacophore

composed of basic amino acids such as lysine, arginine or histidine (8). The presence of hydrophobic amino acids and conjugated fatty acids allows lipopeptides to form an amphipathic structure upon interaction with microbial membranes accounting for their detergent-like properties (9). Ultrashort peptides by definition consist of a maximum of seven amino acid moieties within the primary amino acid sequence (10). Larger molecular weight lipopeptides already exist as licensed therapeutics, including polymyxin B, polymyxin E (colistin) and daptomycin. Lipopeptides themselves are also produced naturally by bacteria and fungi to provide competitive advantage against competing microbes (11). The prospect exists of tailoring the amino acid and lipophilic structure to vary the spectrum of activity and selectivity of these ultrashort lipopeptides within a minimum structural motif.

Previous results from our group showed that conjugation of dodecanoic acid (C₁₂) to the tetrapeptide amide motif amide H-OOWW-NH₂, described previously by Bisht and colleagues (12), displayed potent biofilm eradication properties in the µg/mL range, with a MBEC value as low as 15.63 µg/mL against 24 hour biofilms of methicillin-resistant *Staphylococcus epidermidis* (MRSE) (ATCC 35984) (13). Increased selection for biofilm cells relative to mammalian cells was displayed over similar concentration range *in vitro*. We were also able to incorporate these ultrashort cationic peptides and the amphibian antimicrobial peptide Maximin-4 into poly(2-hydroxyethyl methacrylate) hydrogels for potential use in the prevention of biomaterial associated infections (14). These results show promise at a time when modern medicine and society are facing an increasing prevalence of resistant hospital and community acquired infections and a proportionate slowing in the development of innovative antimicrobial compounds (15). Increasing rates of resistant infection can be attributed partly to the ability of pathogens to form biofilms: a thick, extracellular polymeric matrix that surrounds microbial cells when attached to surfaces, such as indwelling medical devices. The biofilm matrix protects microbial cells from antimicrobial attack and provides an optimum environment for survival (16). For antimicrobial treatment strategies to be successful they must take into account the presence of more resistant, sessile biofilm forms of microorganisms. Targeting of biofilms is essential to ensure complete resolution of infection.

Our study demonstrates kill kinetics against more resistant biofilm forms of microorganisms, the representative phenotype over 80% of clinical infections (17). We describe the implementation of a modified MBEC Assay™ to obtain biofilm kill kinetic profiles for the tetrapeptide H-Orn-Orn-Trp-Trp-NH₂. Improved potency was obtained via the conjugation of dodecanoic acid at the ornithine terminus with comparison made to results for planktonic time kill studies. Ultrashort cationic lipopeptides display a similar spectrum of activity against microbial biofilms as naturally occurring antimicrobial peptides, but with a reduced primary sequence, cost and ease of synthesis.

Methods

Materials

Rink amide 4-(2',4'-dimethoxyphenyl-Fmoc-aminomethyl)-phenoxyacetamido-MHBA (MBHA) resin, all 9-fluorenylmethoxy carbonyl (Fmoc) L-amino acids and 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) were obtained from Merck Chemicals Ltd (Nottingham, UK). Dodecanoic (lauric) acid was obtained from Sigma-Aldrich (Dorset, UK). All other reagents/solvents were peptide synthesis grade.

Peptide synthesis

Peptides were synthesized using standard 9-fluorenylmethoxy carbonyl (Fmoc) solid phase protocols on Rink Amide MHBA resin, using a CEM Liberty (Buckingham, UK) microwave enhanced automated peptide synthesizer as previously described (13).

Strains and growth conditions

The following strains were used in this study: MRSE (ATCC 35984), *S.aureus* (ATCC 29213), methicillin-resistant *Staphylococcus aureus* (MRSA) (ATCC 43300), *Pseudomonas aeruginosa* (*P.aeruginosa*) (PA01), *Escherichia coli* (*E.coli*) (NCTC 8196), and *Candida tropicalis*

(*C.tropicalis*) (NCTC 7393). All microbial strains were stored at -70 °C in Microbank vials (Pro-Lab Diagnostics, Cheshire, UK) and subcultured in Müller Hinton Broth (MHB) before testing.

Universal neutralizer formulation

A stock solution of universal neutralizer was formed as demonstrated by Booth and colleagues (2013) (18). This stock solution was stored as aliquots in sterile eppendorfs and frozen at -20°C until required. 500µl of the universal neutralizer was added to 20mLs of 0.9% sodium chloride solution for use in universal inhibitor dilution plates.

Planktonic Kill Kinetics

The ability of the peptides to kill the planktonic form of MRSE (ATCC 35984), *S.aureus* (ATCC 29213), MRSA (ATCC 43300), *Pseudomonas aeruginosa* (PAO1), *E.coli* (NCTC 8196) and *C.tropicalis* (NCTC 7393) were evaluated according to CLSI guidelines (19). Peptides were selected for kinetic evaluation at the minimum bactericidal (MBC) or minimum fungicidal (MFC) concentration previously determined (13). This provided valid analysis of the kill kinetics of each antimicrobial as demonstrated by Stratton and colleagues (20). Time points selected to assess planktonic kill kinetics were 10 minutes (0.1667 hours), 1, 1.5, 2, 4, 6 and 24 hours with quenching of antimicrobial achieved by formulation of a universal neutralizer.

Biofilm Kill Kinetics

Biofilm rate of kill assays were performed using the Calgary Biofilm Device (MBEC Assay™ P&G)(Innovotech Inc., Alberta, Canada), as previously described (21,22). After incubation, mature 24 hour biofilm of each susceptible microorganism was challenged with the MBEC value and 200µg/mL of peptide (if the MBEC value was equal to or lower than 1000µg/mL). No activity corresponds to MBEC value greater than 1000µg/mL, outside the tested concentration range from the results obtained previously by our group (13). A 200µg/mL variable was used to assess how the same concentration would affect the kill kinetics against different

microorganisms. Time points selected for kinetic biofilm eradication assessment were 0.5, 1, 1.5, 2, 3, 4, 6 and 24 hours. At each of these time points 5 pegs were removed, using sterile flamed pliers, from the MBEC lid corresponding to challenged biofilm and a further 5 pegs corresponding to positive controls (phosphate buffered saline [PBS]) for the biofilm and placed into separate wells of a dilution plates containing universal neutralizer. These were serially diluted for subsequent plating on Mueller-Hinton agar to obtain Miles and Misra viable counts for each timepoint.

Statistical Analysis

Statistical analyses were carried out using Microsoft Excel 2010 and GraphPad InStat 3. Standard deviations were obtained for viable counts at each concentration of antimicrobial tested based on 5 replicates for biofilm counts over 24 hours exposure to varying concentrations ($\mu\text{g/mL}$) of ultrashort antimicrobial lipopeptides and five replicates for rate of kill assays. The influence of peptides studied on rate of biofilm eradication (biofilm viable count (Log_{10} CFU/peg) at each time point) and their effect on the planktonic rate of kill (Log_{10} planktonic viable counts (Log_{10} CFU/mL)) were examined using a one way ANOVA, with a Tukey-Kramer multiple comparisons test used to identify individual differences relative to the positive control (PBS) for each peptide at each timepoint. In all cases a probability of $p \leq 0.05$ denoted significance.

Results and Discussion

Cationic antimicrobial peptides and particularly the lipopeptide varieties are of huge importance in combating resistance to standard antibiotics as they act rapidly to fully eradicate biofilm, unlike some standardly employed antimicrobial regimens (23). Against the less resistant planktonic phenotype rapid bactericidal action for all peptides was achieved against all microbial isolates (Figures 1-6) within 4 hours at respective MBC or MFCs. Activity was particularly rapid against Gram-positive bacterial isolates with 100% kill for the ultrashort lipopeptide C₁₂-

OOWW-NH₂ obtained within 1 hour for MRSE (ATCC 35984), *S.aureus* (ATCC 29213) and MRSA (ATCC 43300).

Increased tolerance to antimicrobial peptides is displayed by the biofilm phenotype compared to the planktonic forms due to the presence of an exopolysaccharide matrix with the ability to physically slow the diffusion of antimicrobials through the biofilm (24). Nevertheless the peptides tested showed rapid action against a range of biofilm cells, with the potential for resistance development against lipopeptides in particular, reduced due to complete biofilm kill at low exposure times. Complete eradication (5.55 Log₁₀ CFU/peg reduction, P<0.001) of 24 hour established biofilms of MRSE (ATCC 35984) at the MBEC (15.63µg/ml) of C₁₂-OOWW-NH₂ was achieved within 4 hours (Figure 7). An increase of almost thirteen times this concentration (from 15.63µg/ml to 200µg/ml) resulted in a significant reduction within 1 hour (4.9 Log₁₀ CFU/peg, P<0.001) and complete eradication within 2 hours, thus demonstrating the dose-dependent activity of these peptides. The significant (P<0.001) rapid reduction in microbial viability demonstrated by C₁₂-OOWW-NH₂ and Maximin-4 against planktonic and biofilm phenotypes of MRSE within 1 hour, utilizing our kill kinetics model, correlates with a significant reduction in the adherence of MRSE within 1 hour using our *in vitro* biomaterial model with these peptides (14). These results are also replicated for C₁₂-OOWW-NH₂ when tested against all Gram-positive bacteria tested (methicillin sensitive and resistant *S.aureus*, Figures 8 and 9 respectively). Kill kinetics were improved compared with non-lipidated H-OOWW-NH₂. Previous work by Smith and colleagues demonstrated that the licensed antimicrobials clindamycin, daptomycin, linezolid, tigecycline and vancomycin were unable to completely kill biofilms of MRSA with mean cell survival in treated biofilms of 62%, 4%, 45%, 43% and 19% respectively after 24 hours using an XTT assay (22). Therefore the rapid rate of biofilm kill achieved by C₁₂-OOWW-NH₂ is even more significant when compared with the activity of antibacterials such as linezolid and vancomycin, which are reserved for cases of more resistant infections (25).

Biofilms of *Pseudomonas aeruginosa* did not exhibit complete eradication in the presence of any of the peptides/lipopeptides tested within 24 hours; up to a concentration of 1000µg/ml. Therefore kill kinetic analyses were not performed on the biofilm phenotype. *E.coli* biofilms (Figure 10) were only completely eradicated within 24 hours by C₁₂-OOOWW-NH₂ at a concentration of 500µg/ml (a thirty two fold increase in MBEC compared with MRSE and an eight fold increase compared with MRSA). Significant reduction (3 Log₁₀ CFU/peg reduction, P<0.001) was achieved within 1 hour exposure to the higher 500µg/ml MBEC value. Fungal *C.tropicalis* biofilms (Figure 11) were eradicated by C₁₂-OOWW-NH₂ within 24 hours at concentrations of 250µg/ml (MBEC), however significant reduction (P<0.001) in viable biofilm was achieved at both 200µg/ml (3.59 Log₁₀ CFU/peg reduction) and 250µg/ml (3.83 Log₁₀ CFU/peg reduction) within 4 hours exposure. Biofilm eradication occurs within 24 hours at MBEC for C₁₂-OOWW-NH₂ against *E.coli* (NCTC 8196) and *C.tropicalis* (NCTC 7393) with reduced kill kinetics. C₁₂-OOOWW-NH₂ displayed a broader range of antimicrobial activity than the amphibian-derived peptide Maximin-4 and with the added benefit of reduced synthesis costs and relative ease of synthetic manufacture.

Despite the differences in antimicrobial potencies demonstrated by the ultrashort cationic peptides and Maximin-4 they share similar antimicrobial activity with regard to their mechanism of action. Targeting of microbial membranes is likely to account for the rapid bactericidal and biofilm eradication obtained in this report. These cationic peptides bind preferentially to the phospholipid bilayer of bacterial cells, which has an overall negative charge compared to neutral mammalian cells (26) due to the presence of acidic hydroxylated phospholipids, such as phosphatidylglycerol, cardiolipin and phosphatidylserine in the latter (4). Additional acidic polymers, such as teichoic acids specific to Gram-positive bacteria (27) and phosphate groups present on lipopolysaccharides in Gram-negative bacteria (28), aid in the initial attachment of the peptide. This is prior to the formation of transmembrane pores, resulting in self-promoted uptake and membrane permeabilization via the toroidal pore, aggregate, barrel stave, and carpet models (9). Cationic antimicrobial peptides possess both hydrophobic and hydrophilic amino acid residues that allow disruption of bacterial membranes. Improved kill kinetics and reduced MBCs, MFC and MBECs are achieved for C₁₂-OOOWW-NH₂ relative to non-lipidated H-OOWW-NH₂

and Maximin-4 as this primary structure has the most optimal hydrophobic: hydrophilic balance, thus ensuring increased interaction with microbial membranes and maximal antimicrobial activity (9,13). As previously reported by our group haemolysis and cell cytotoxicity studies indicated that lipopeptides based on the C_n-OOWW-NH₂ motif (where C_n represents the number of carbons on the terminal acyl grouping) displayed reduced mammalian cell cytotoxicity and increased selectivity for microbial membranes, via comparison with respective MBEC values. They have the potential to be safely used within the MIC values for all microorganisms tested (13).

Binding of the positively charged peptides to negatively charged biofilm polymeric materials of Gram-negative bacteria may retard the penetration of these antimicrobial peptides through the biofilm, thus explaining a relative lack of activity against *Pseudomonas aeruginosa* and *E.coli* biofilms. This effect has been described previously in relation to positively charged aminoglycosides and *Pseudomonas* biofilms (16) and the antiseptic, chlorhexidine and oral biofilms (29). The activity of cationic antimicrobial peptides are also affected by the Gram-negative PhoP/PhoQ regulon and the PmrA/PmrB system, that result in the addition of aminoarabinose to lipid A present in the outer membrane of Gram-negative bacteria. This creates a membrane with increased cationicity and greater resistance to cationic antimicrobial peptides (30). However, membrane disruption may allow synergism with a standard antimicrobial that targets microbial cell metabolism, for example rifampicin, which can enter the cell in higher quantities to exert its antimicrobial effect. The self-promoted uptake model provides an explanation as to why in Gram-negative bacteria many cationic antimicrobial peptides act in synergy with conventional antibiotics (31). This strategy may serve as a viable resolution for improving the activity of the C₁₂-OOOWW-NH₂ in Gram-negative biofilms which proved difficult to eradicate fully in our investigations. Synergy of cationic antimicrobial peptides with standard antibiotics is not limited to just Gram-negative bacteria but has been proven for both Gram-positive bacteria (32) and fungi (33). Clinically such a strategy has been employed successfully with the use of the licensed lipopeptide colistin. Combination therapy with tobramycin has shown to be superior to single antibiotic monotherapy with the same drugs in the treatment of cystic fibrosis patients infected with *Pseudomonas aeruginosa* (34). The use of

ultrashort lipopeptides may serve as a viable alternative to colistin and other standardly used antimicrobials for a range of biofilm related infections, with extensive *in vivo* analysis and pharmaceutical formulation studies of C₁₂-OOOWW-NH₂ required to study its true clinical potential.

Conflicts of Interest

The authors declare no conflict of interest.

FigureS

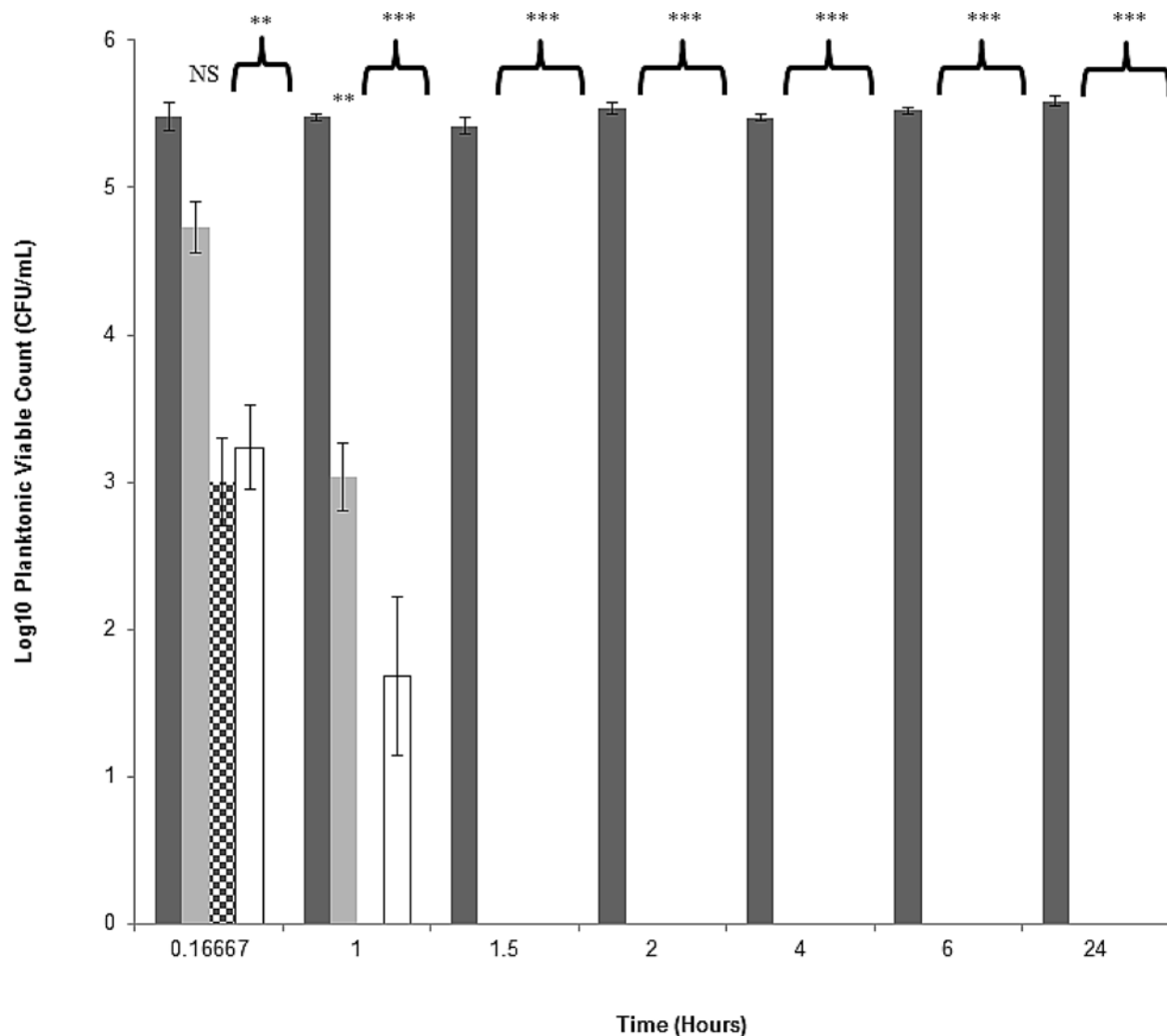


Fig. 1 Log₁₀ planktonic viable counts (Log₁₀CFU/mL) of MRSE (ATCC 35984) over a period of 24 hour exposure to the respective MBC value (µg/mL) of H-OOWW-NH₂, C₁₂-OOWW-NH₂ and Maximin-4 based on five replicates.

Key:

■ Positive control ■ H-OOWW-NH₂: 250µg/ml ▨ C12-OOWW-NH₂: 1.95µg/ml □ Maximin-4: 15.63µg/ml

NS: no significant difference ($P > 0.05$), *: $P < 0.05$, **: $P < 0.01$, ***: $P < 0.001$, significant difference between Log₁₀CFU/mL of peptide and the positive control at the same time point

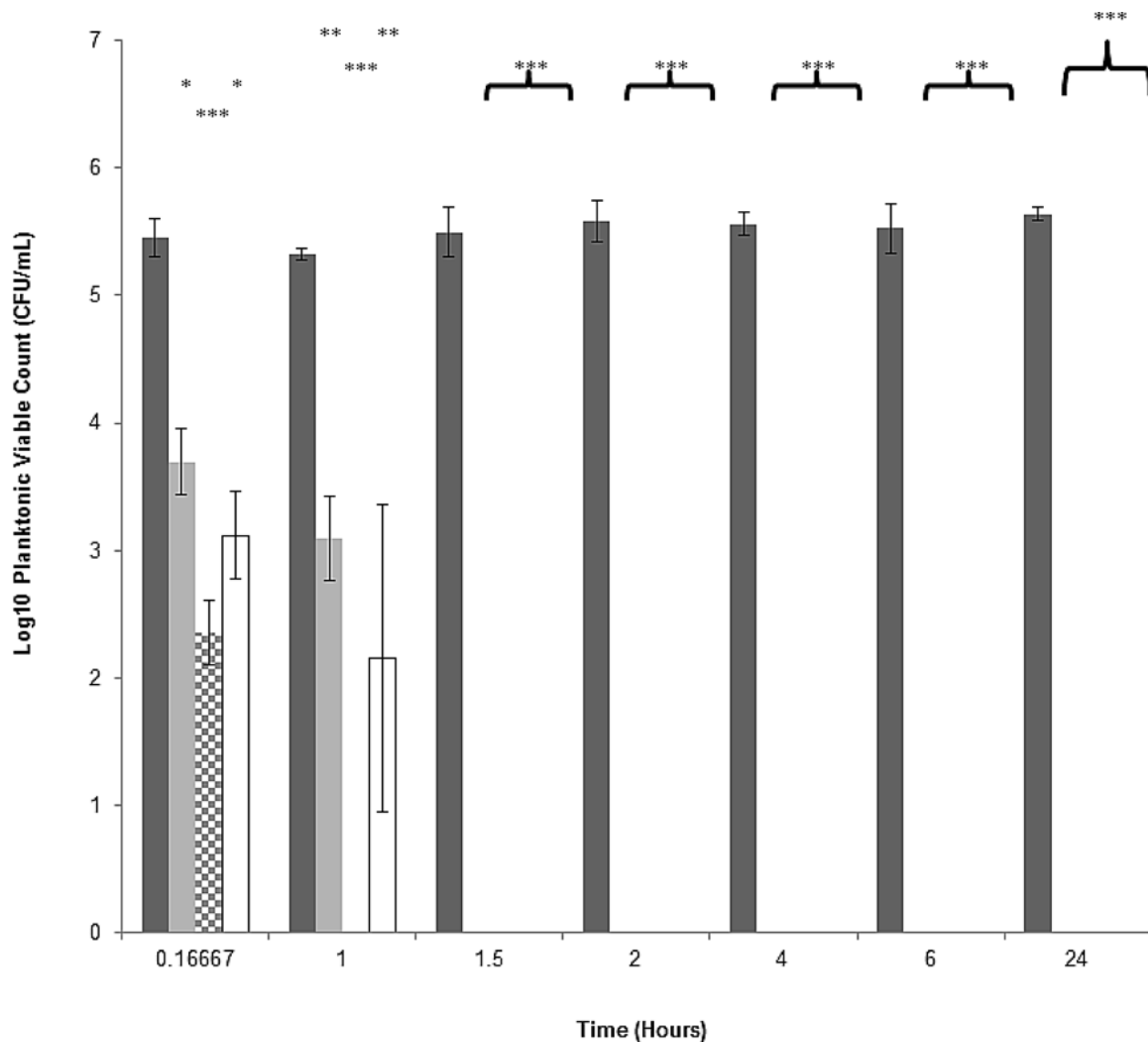


Fig. 2 Log₁₀ planktonic viable counts (Log₁₀CFU/mL) of *S.aureus* (ATCC 29213) over a period of 24 hour exposure to the respective MBC value (μg/mL) of H-OOWW-NH₂, C₁₂-OOWW-NH₂ and Maximin-4 based on five replicates.

Key:

■ Positive control ■ H-OOWW-NH₂: 250 μg/mL ▨ C12-OOWW-NH₂: 1.95 μg/mL □ Maximin-4: 15.63 μg/mL

NS: no significant difference (P>0.05), *: P<0.05, **: P<0.01, ***: P<0.001, significant difference between Log₁₀CFU/mL of peptide and the positive control at the same time point

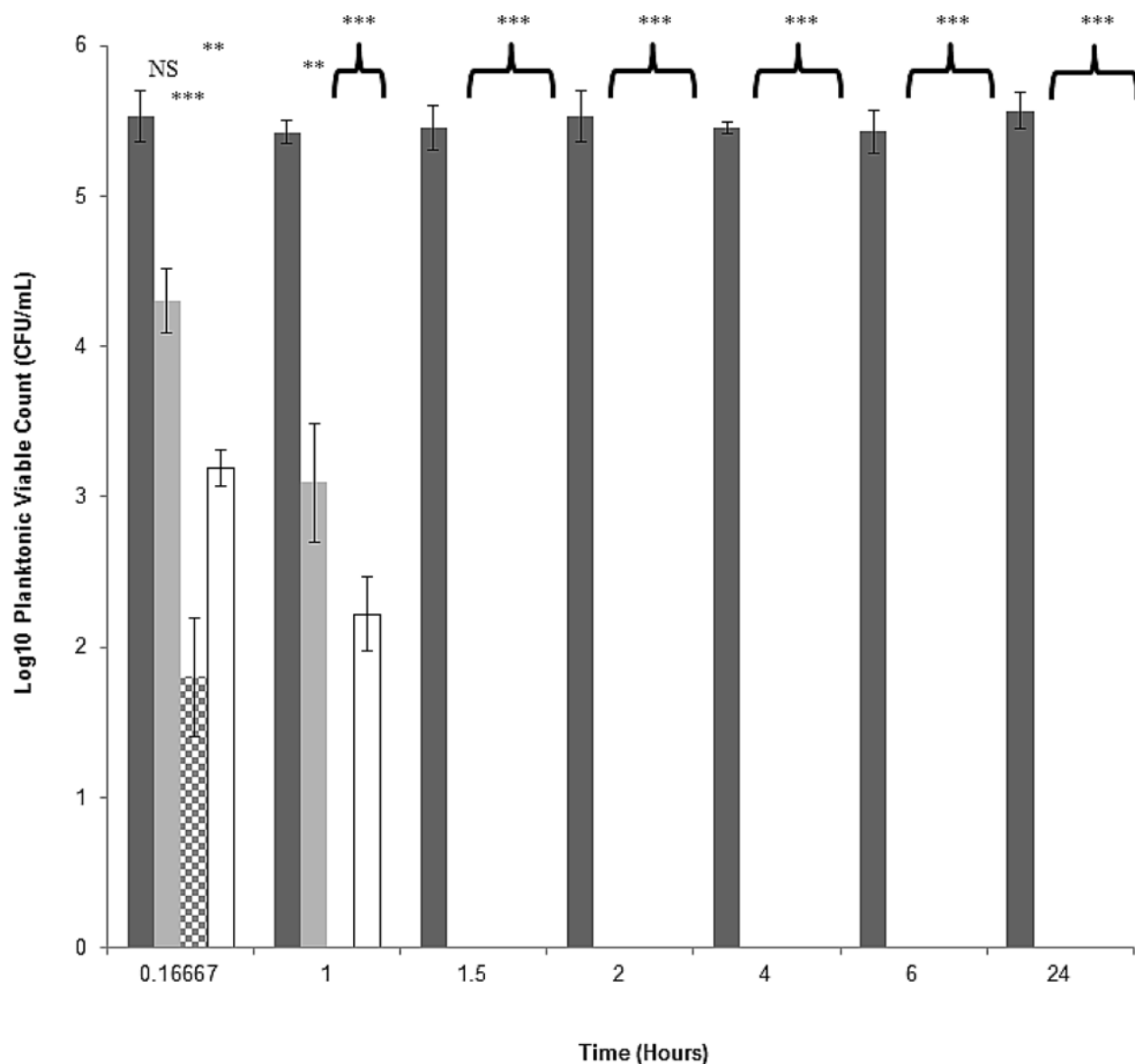


Fig. 3 Log₁₀ planktonic viable counts (Log₁₀CFU/mL) of MRSA (ATCC 43300) over a period of 24 hour exposure to the respective MBC value (μg/mL) of H-OOWW-NH₂, C₁₂-OOWW-NH₂ and Maximin-4 based on five replicates.

Key:

■ Positive control ■ H-OOWW-NH₂: 250μg/ml ▨ C12-OOWW-NH₂: 3.91μg/ml □ Maximin-4: 15.63μg/ml

NS: no significant difference (P>0.05), *: P<0.05, **: P<0.01, ***: P<0.001, significant difference between Log₁₀CFU/mL of peptide and the positive control at the same time point

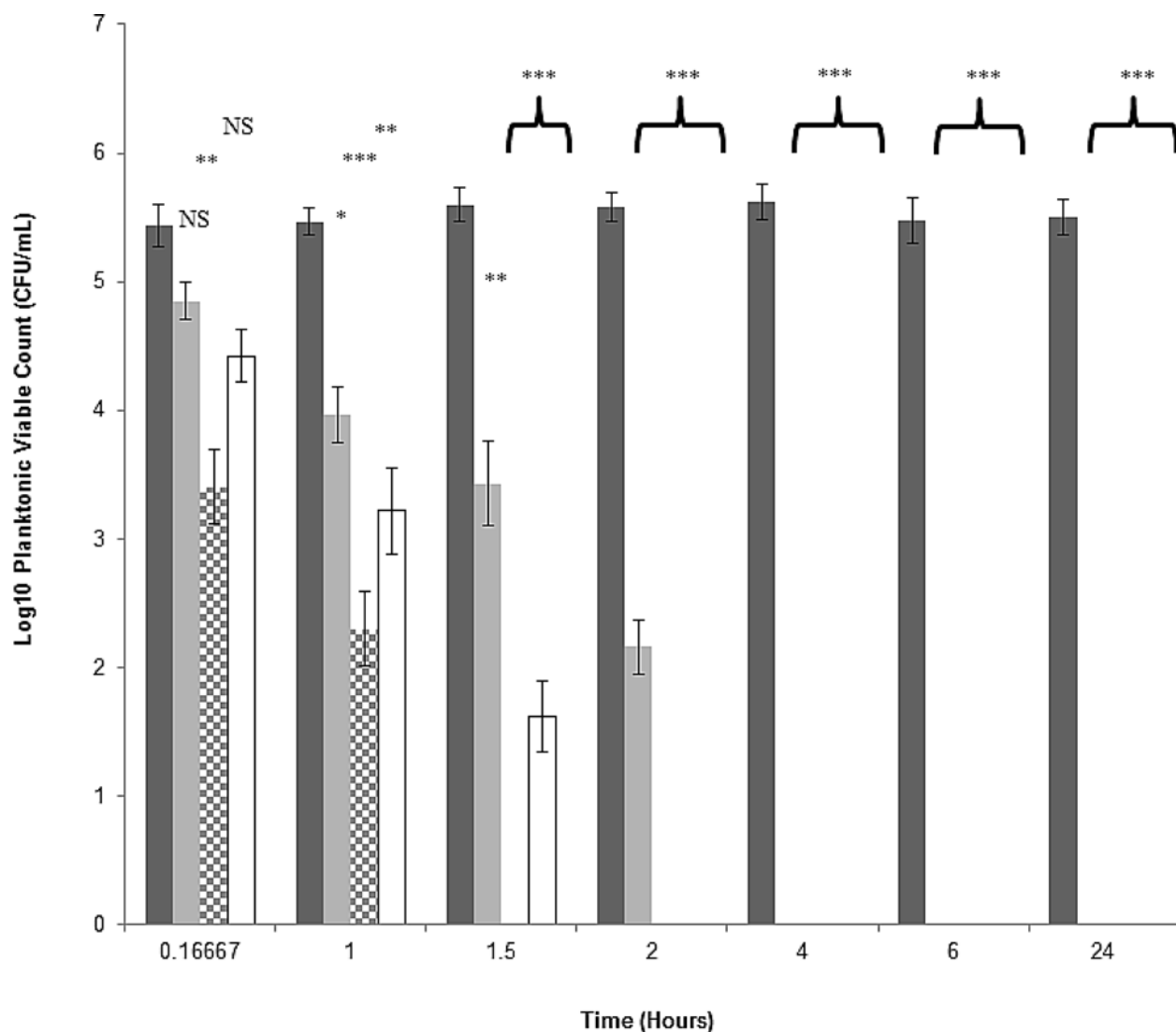


Fig. 4 Log₁₀ planktonic viable counts (Log₁₀CFU/mL) of *Pseudomonas aeruginosa* (PAO1) over a period of 24 hour exposure to the respective MBC value (µg/mL) of H-OOWW-NH₂, C₁₂-OOWW-NH₂ and Maximin-4 based on five replicates.

Key:

■ Positive control ■ H-OOWW-NH₂: 500µg/ml × C12-OOWW-NH₂: 15.63µg/ml □ Maximin-4: 31.25µg/ml

NS: no significant difference (P>0.05), *: P<0.05, **: P<0.01, ***: P<0.001, significant difference between Log₁₀CFU/mL of peptide and the positive control at the same time point

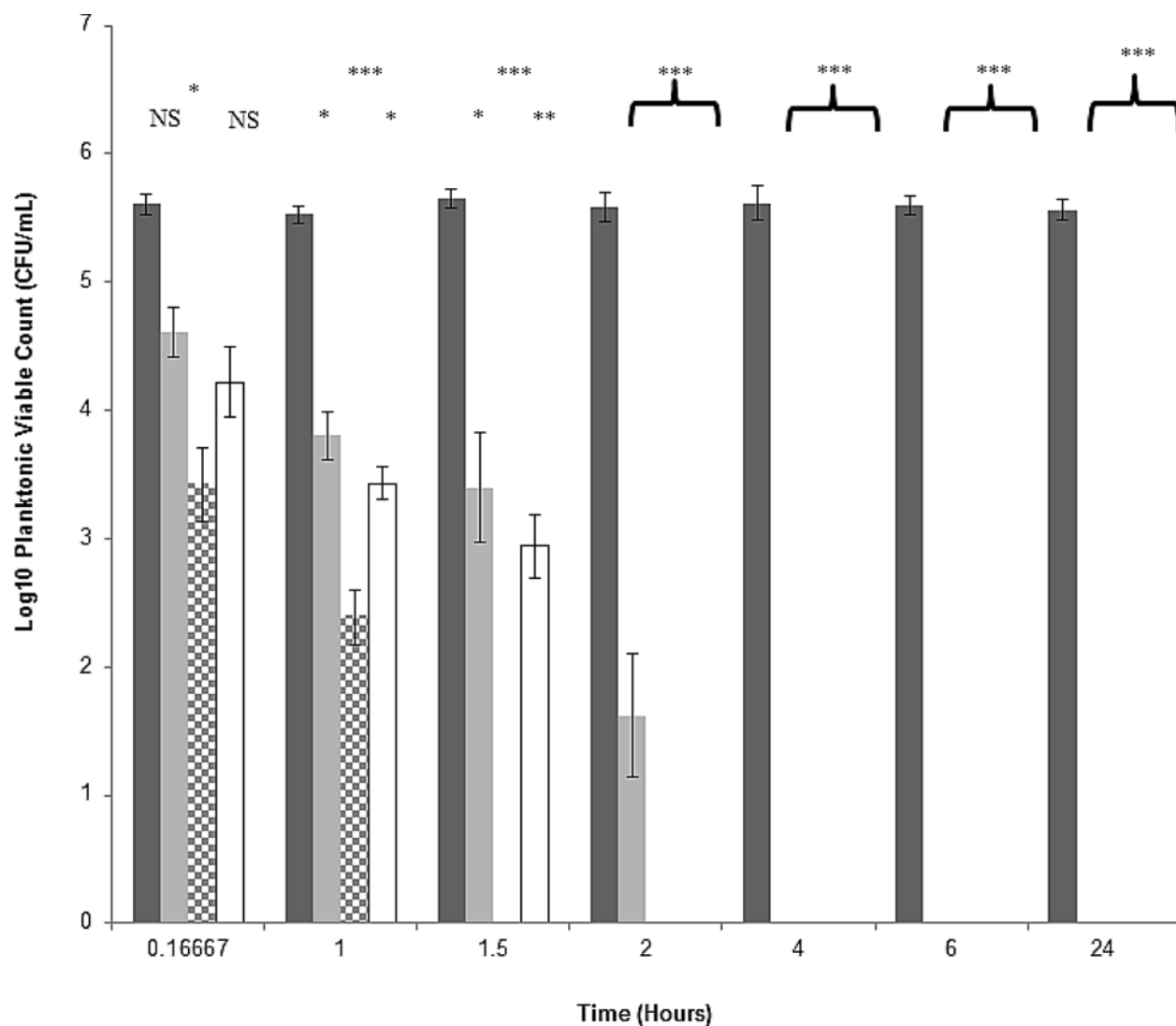


Fig. 5 Log₁₀ planktonic viable counts (Log₁₀CFU/mL) of *E.coli* (NCTC 8196) over a period of 24 hour exposure to the respective MBC value (μg/mL) of H-OOWW-NH₂, C₁₂-OOWW-NH₂ and Maximin-4 based on five replicates.

Key:

■ Positive control ■ H-OOWW-NH₂: 500μg/ml ▨ C12-OOWW-NH₂: 15.63μg/ml □ Maximin-4: 250μg/ml

NS: no significant difference (P>0.05), *: P<0.05, **: P<0.01, ***: P<0.001, significant difference between Log₁₀CFU/mL of peptide and the positive control at the same time point

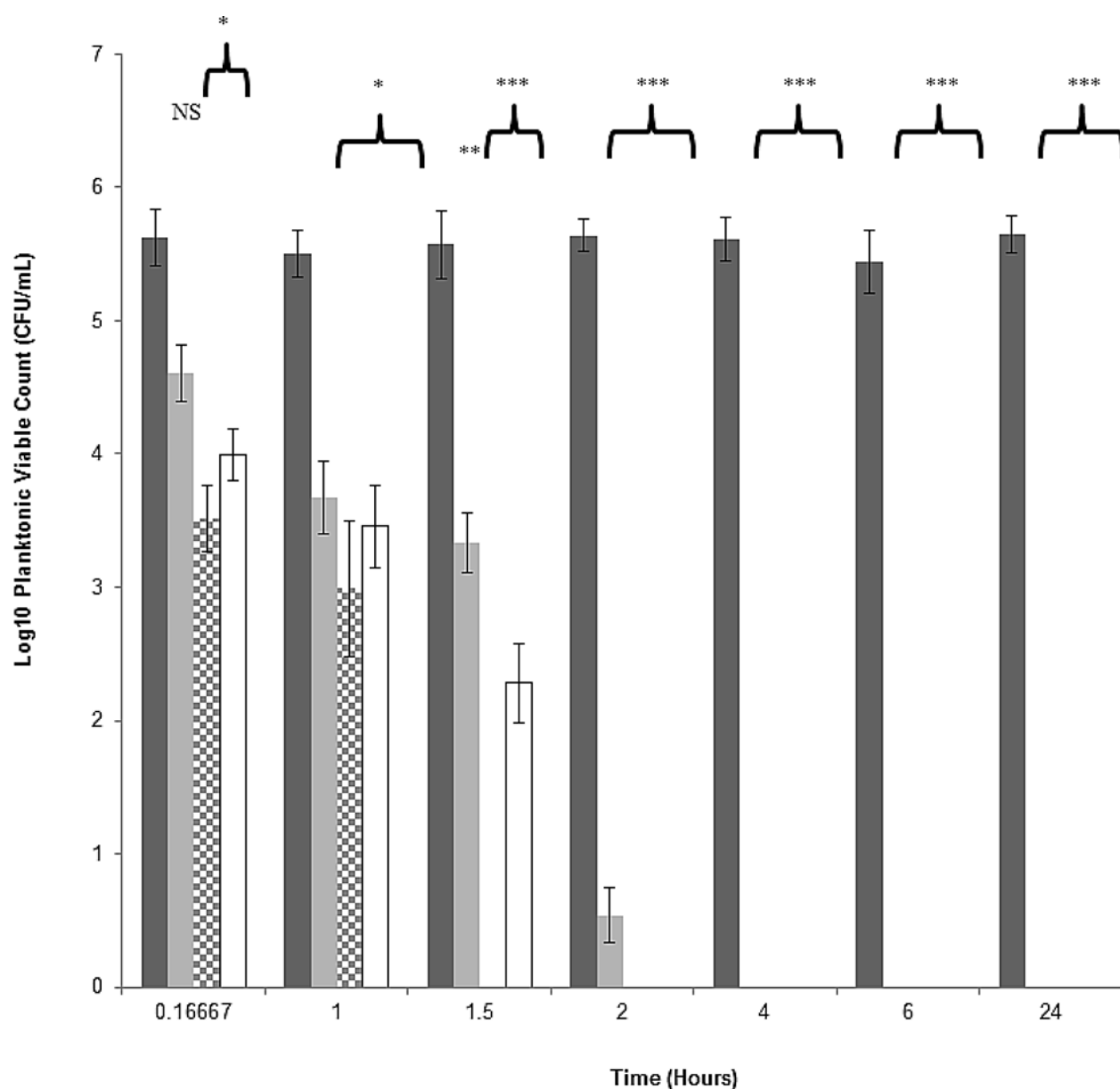


Fig. 6 Log₁₀ planktonic viable counts (Log₁₀CFU/mL) of *C.tropicalis* (NCTC 7393) over a period of 24 hour exposure to the respective MFC value (µg/mL) of H-OOWW-NH₂, C₁₂-OOWW-NH₂ and Maximin-4 based on five replicates.

Key:

■ Positive control ■ H-OOWW-NH₂: 250µg/ml ▨ C12-OOWW-NH₂: 3.91µg/ml □ Maximin-4: 1.95µg/ml

NS: no significant difference (P>0.05), *: P<0.05, **: P<0.01, ***: P<0.001, significant difference between Log₁₀CFU/mL of peptide and the positive control at the same time point

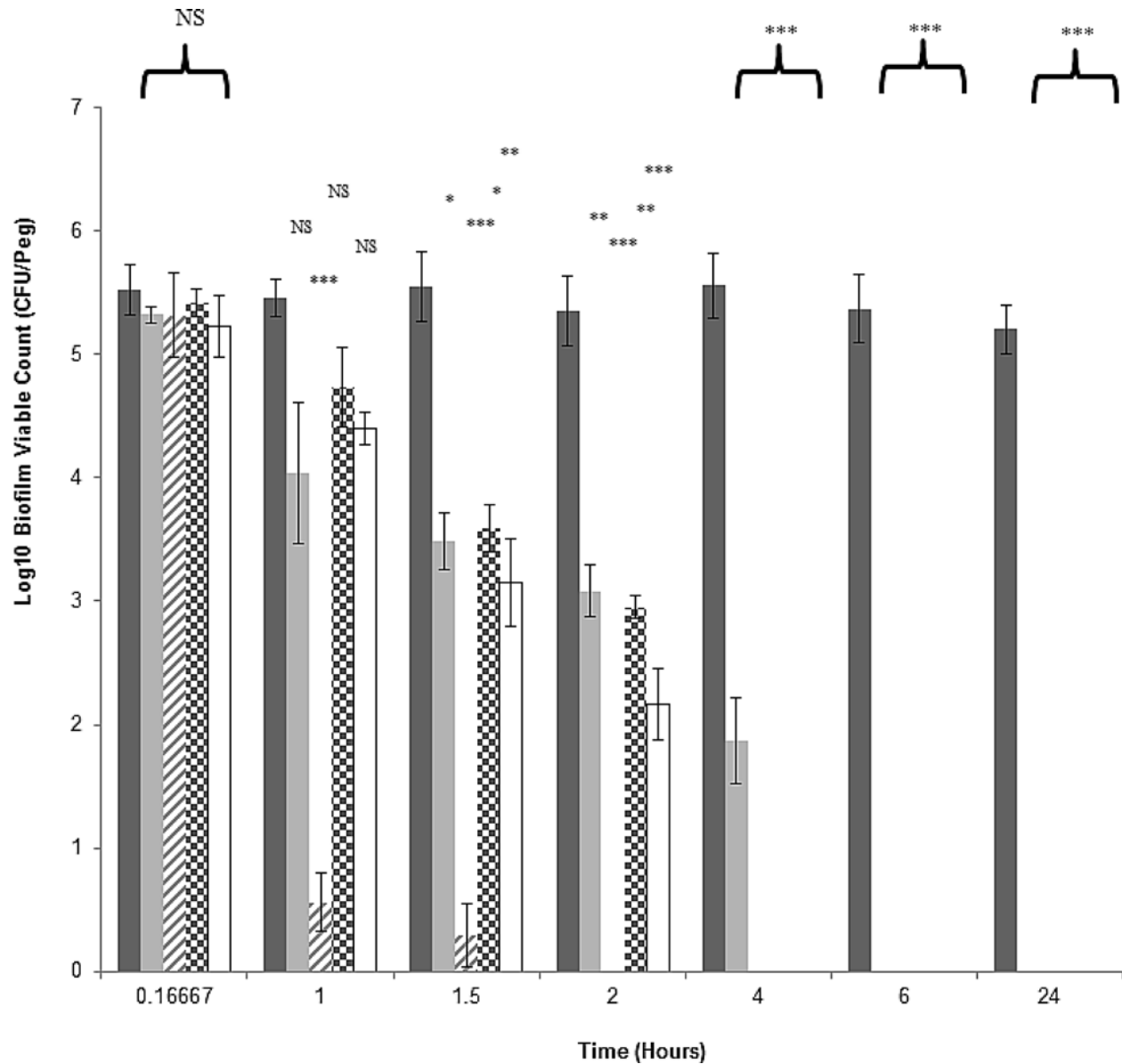


Fig. 7 Log₁₀ biofilm viable counts (Log₁₀CFU/peg) of MRSE (ATCC 35984) over a period of 24 hour exposure to the respective MBEC value (µg/mL) of H-OOWW-NH₂, C₁₂-OOWW-NH₂ and Maximin-4 based on five replicates. C₁₂-OOWW-NH₂ was also tested at 200µg/mL as a relative comparator to the MBEC for ultrashort lipopeptides.

Key:

- Positive control
- H-OOWW-NH₂: 500µg/ml
- ▨ C12-OOWW-NH₂: 200µg/ml
- ▧ C12-OOWW-NH₂: 15.63µg/ml
- Maximin-4: 31.25µg/ml

NS: no significant difference ($P>0.05$), *: $P<0.05$, **: $P<0.01$, ***: $P<0.001$, significant difference between $\text{Log}_{10}\text{CFU/mL}$ of peptide and the positive control at the same time point

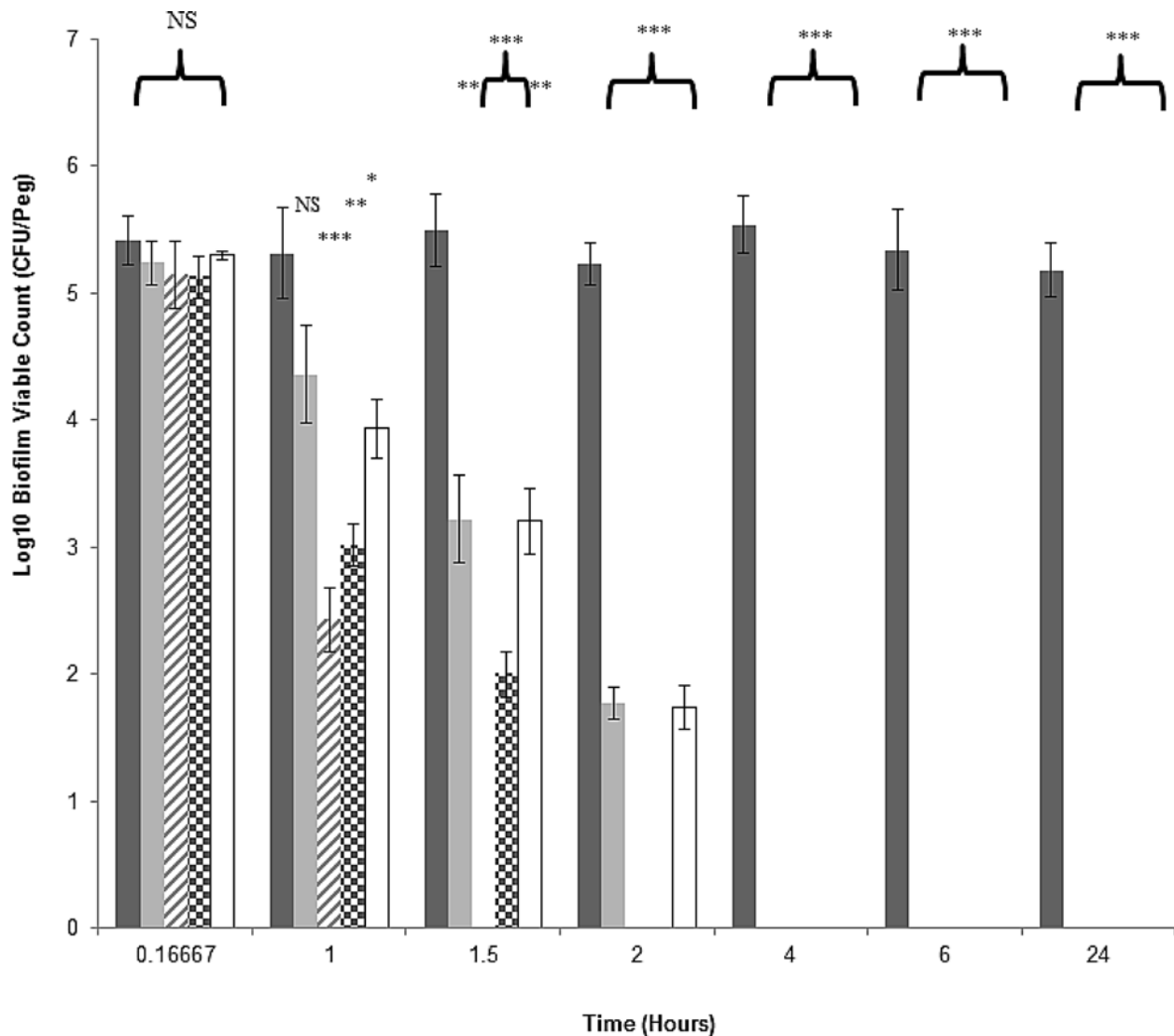


Fig. 8 Log_{10} biofilm viable counts ($\text{Log}_{10}\text{CFU/peg}$) of *S.aureus* (ATCC 29213) over a period of 24 hour exposure to the respective MBEC value ($\mu\text{g/mL}$) of H-OOWW-NH₂, C₁₂-OOWW-NH₂ and Maximin-4 based on five replicates. C₁₂-OOWW-NH₂ was also tested at 200 $\mu\text{g/mL}$ as a relative comparator to the MBEC for ultrashort lipopeptides.

Key:

■ Positive control ■ H-OOWW-NH2: 500µg/ml ▨ C12-OOWW-NH2: 200µg/ml
 ▩ C12-OOWW-NH2: 62.5µg/ml □ Maximin-4: 15.63µg/ml

NS: no significant difference ($P>0.05$), *: $P<0.05$, **: $P<0.01$, ***: $P<0.001$, significant difference between $\text{Log}_{10}\text{CFU/mL}$ of peptide and the positive control at the same time point

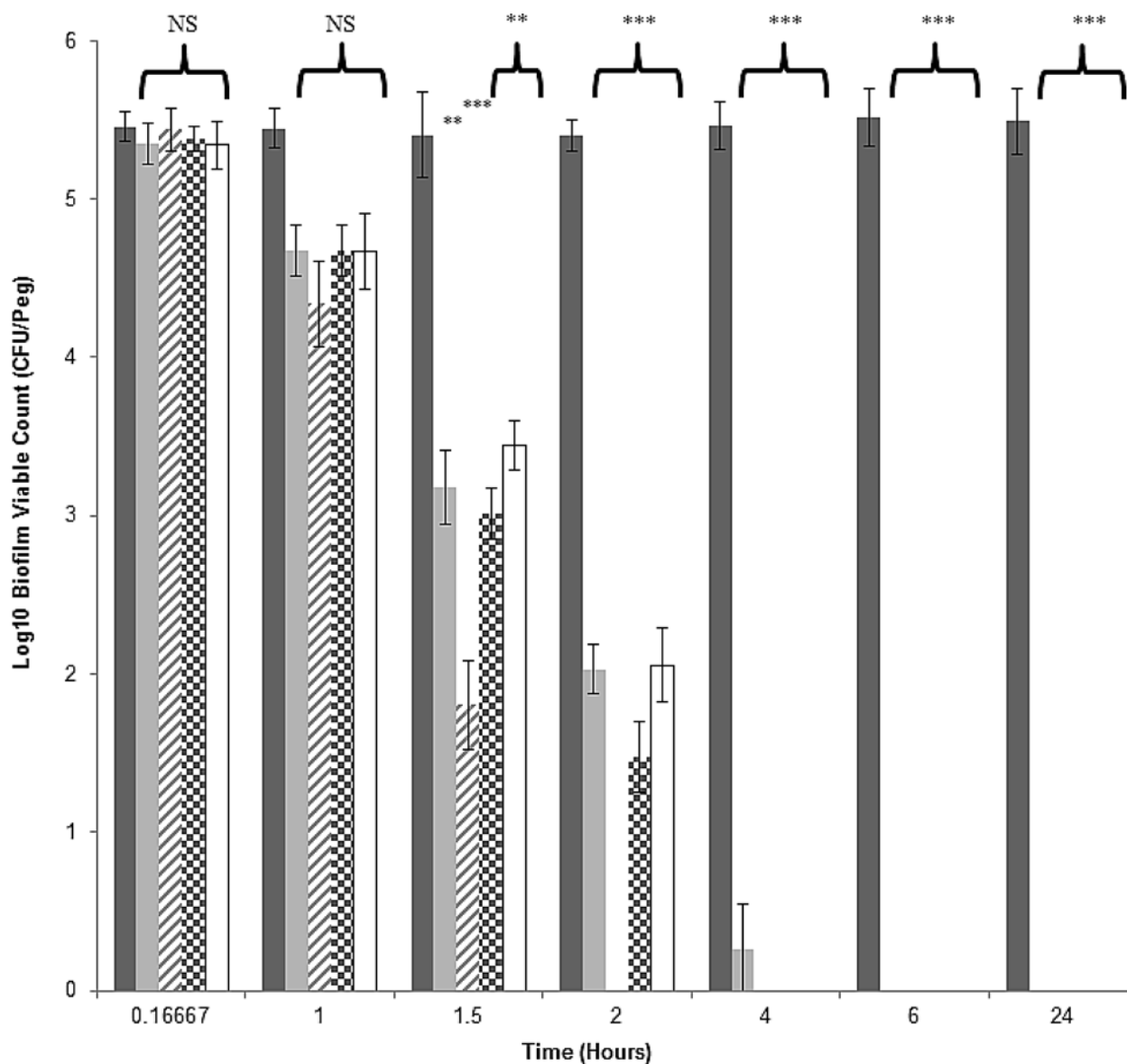


Fig. 9 Log_{10} biofilm viable counts ($\text{Log}_{10}\text{CFU/peg}$) of MRSA (ATCC 43300) over a period of

24 hour exposure to the respective MBEC value ($\mu\text{g/mL}$) of H-OOWW-NH₂, C₁₂-OOWW-NH₂ and Maximin-4 based on five replicates. C₁₂-OOWW-NH₂ was also tested at 200 $\mu\text{g/mL}$ as a relative comparator to the MBEC for ultrashort lipopeptides.

Key:

■ Positive control ■ H-OOWW-NH₂: 500 $\mu\text{g/mL}$ ▽ C₁₂-OOWW-NH₂: 200 $\mu\text{g/mL}$
▹ C₁₂-OOWW-NH₂: 62.5 $\mu\text{g/mL}$ □ Maximin-4: 250 $\mu\text{g/mL}$

NS: no significant difference ($P>0.05$), *: $P<0.05$, **: $P<0.01$, ***: $P<0.001$, significant difference between Log₁₀CFU/mL of peptide and the positive control at the same time point

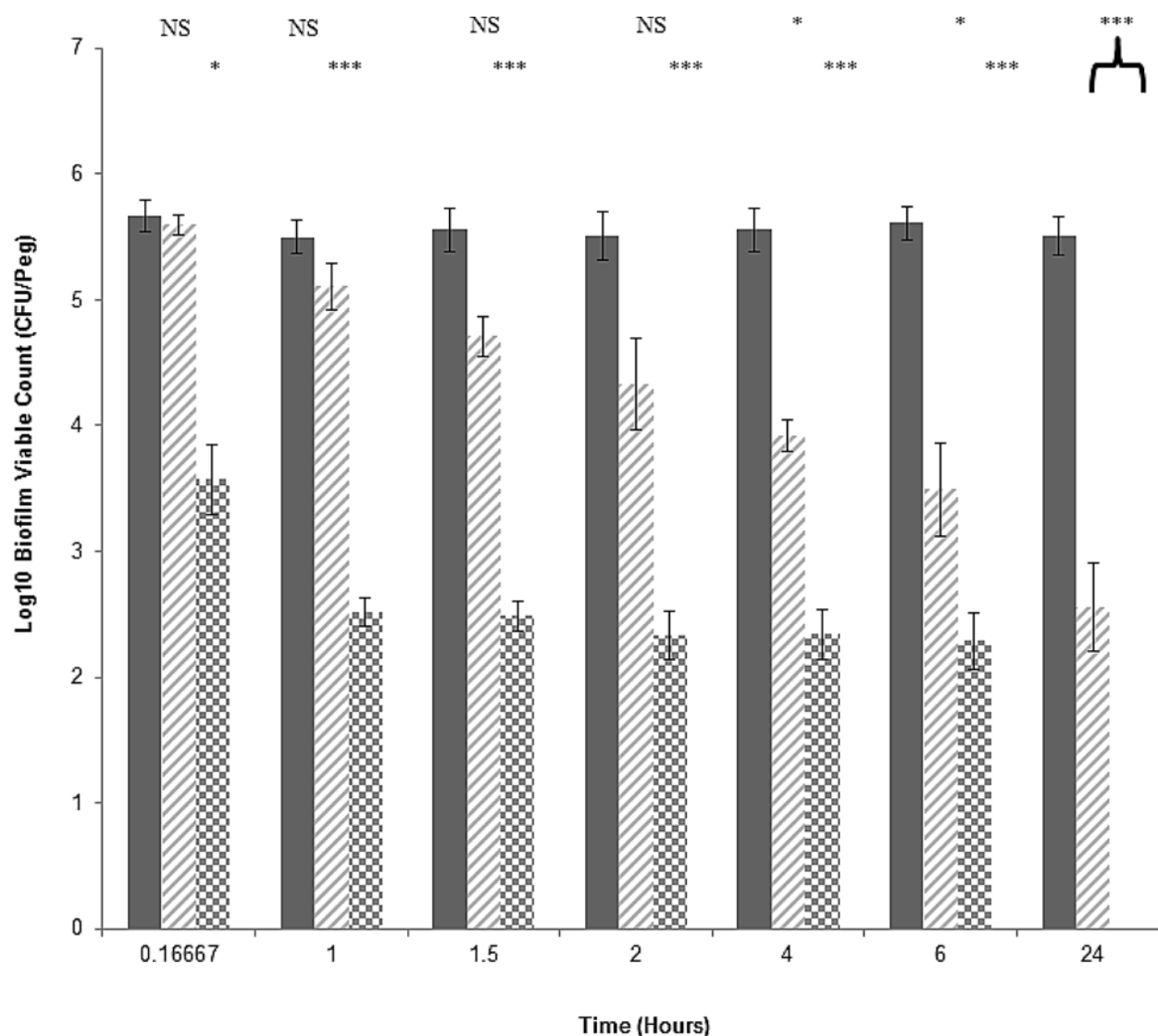


Fig. 10 Log₁₀ biofilm viable counts (Log₁₀CFU/peg) of *E.coli* (NCTC 8196) over a period of 24 hour exposure to the respective MBEC value (µg/mL) of C₁₂-OOWW-NH₂ based on five replicates. C₁₂-OOWW-NH₂ was also tested at 200µg/mL as a relative comparator to the MBEC for ultrashort lipopeptides.

Key:

■ Positive control ▨ C12-OOWW-NH₂: 200µg/ml ▩ C12-OOWW-NH₂: 500µg/ml

NS: no significant difference (P>0.05), *: P<0.05, **: P<0.01, ***: P<0.001, significant difference between Log₁₀CFU/mL of peptide and the positive control at the same time point

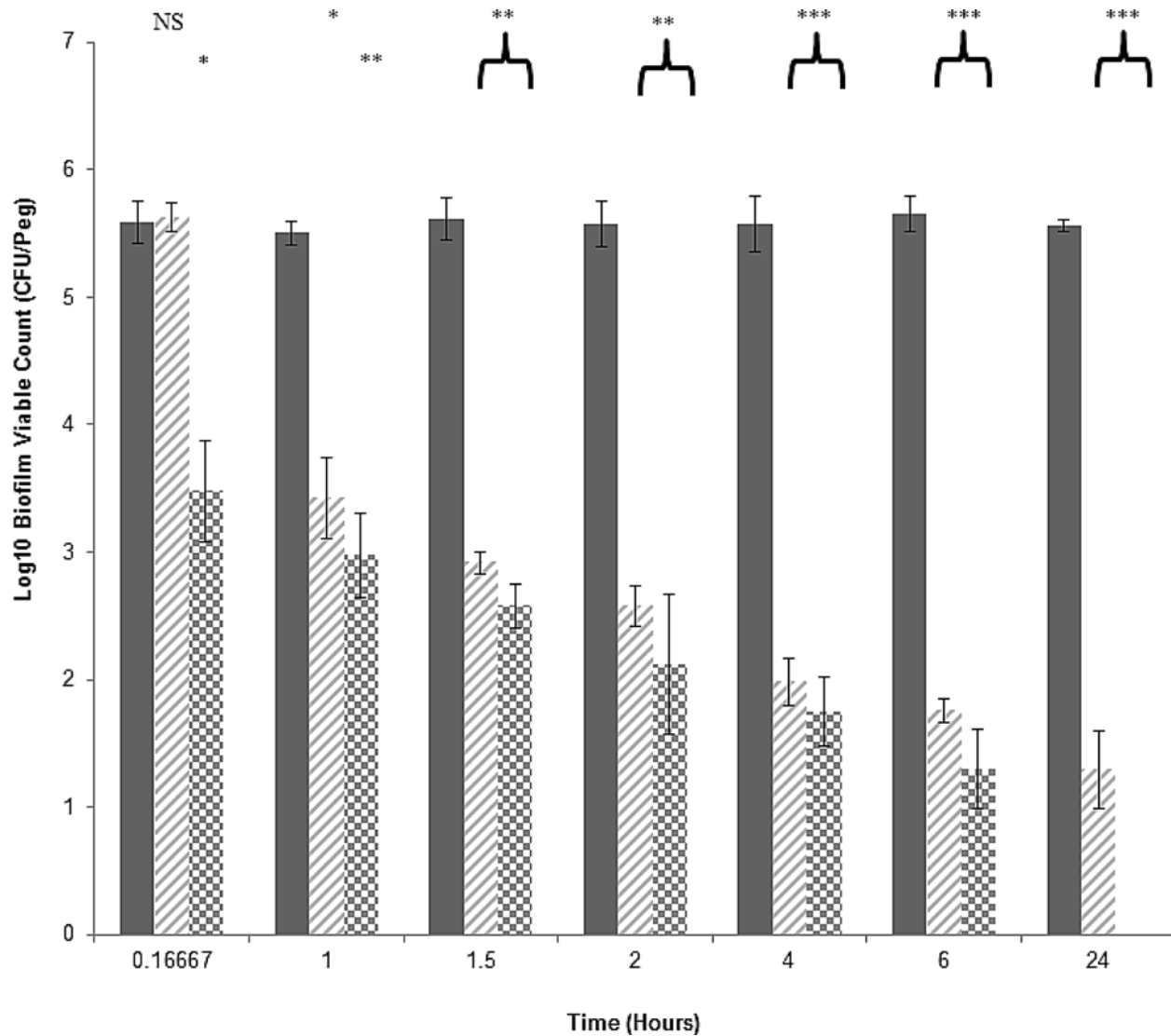


Fig. 11. Log₁₀ biofilm viable counts (Log₁₀CFU/peg) of *C.tropicalis* (NCTC 7393) over a period of 24 hour exposure to the respective MBEC value (µg/mL) of C₁₂-OOWW-NH₂ based on five replicates. C₁₂-OOWW-NH₂ was also tested at 200µg/mL as a relative comparator to the MBEC for ultrashort lipopeptides.

Key:

■ Positive control ▨ C12-OOWW-NH₂: 200µg/ml ▩ C12-OOWW-NH₂: 250µg/ml

NS: no significant difference ($P>0.05$), *: $P<0.05$, **: $P<0.01$, ***: $P<0.001$, significant difference between $\text{Log}_{10}\text{CFU/mL}$ of peptide and the positive control at the same time point

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